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INDIVIDUAL VARIATION IN ASSOCIATIVE LEARNING OF THE NUDIBRANCH MOLLUSC *HERMISSENDA CRASSICORNIS*

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Abstract

Retention of learned suppression of positive phototaxis in the nudibranch mollusc *Hermissenda crassicornis*, induced by exposure to trials of paired light and rotation, was determined for individuals within groups trained in two, three, four, and six daily sessions of 100 trials each. Significant increases in latency to light (acquisition) were measured within all paired treatment groups when these were tested before treatment and 24 hours after the last session. No significant differences in latency were found within four unpaired and one random control group. Next, retention of phototactic suppression (increased latency to respond to light) for each individual was assessed by comparing its post-treatment suppression ratio (SR) scores to a population median score derived from the frequency distribution of scores from a naive group of animals repeatedly tested over a 31-day period. Retention, defined as the consecutive number of days post-treatment on which an animal's SR scores were suppressed below the population median score, was significantly longer in groups trained four and six days than in the two- and three-day paired treatment groups. When retention day score distributions from paired groups were compared to those from the unpaired and random control groups, a significant increase in phototactic suppression was found only for groups trained four and six days. Maximum retention, or resistance to extinction, was measured at 17–18 days (one animal) after 6 sessions. All paired treatments contained animals which did not acquire the association. Retention increased with experience (number of sessions) and the number of animals per group which showed no acquisition decreased.

Investigations on the neural correlates of this behavioral change in *Hermissenda* are currently in progress; an understanding of the relationship between the degree of phototactic suppression in a sample of animals and the number of training sessions will aid in design and interpretation of experiments in which biophysical and biochemical data are correlated with behavioral measures.

Introduction

Interest in the learning abilities of gastropod molluscs has been stimulated by the discovery that these relatively simple animals provide useful models for studies on the neuronal basis of learning. Associative learning has now been studied in five gastropod species (Mpitsos and Davis, 1973; Alkon, 1974; Gelperin, 1975; Crow and Alkon, 1978; Walters *et al.*, 1979; Audesirk *et al.*, 1982). Behavioral acts modified by conditioning procedures include: (1) feeding behavior—*Limax maximus*

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(Gelperin, 1975; Sahley *et al.*, 1981), *Pleurobranchaea californica* (Mpitsos and Davis, 1973; Mpitsos and Collins, 1975; Davis *et al.*, 1980), *Lymnaea stagnalis* (Alexander *et al.*, 1982, 1984; Audesirk *et al.*, 1982); (2) escape-withdrawal locomotion—*Aplysia californica Pleurobranchaea* (Mpitsos and Collins, 1975; Walters *et al.*, 1979; Carew *et al.*, 1981, 1983); and (3) positive phototaxis—*Hermissenda crassicornis* (Alkon, 1974; Crow and Alkon, 1978; Crow and Harrigan, 1989; Farley and Alkon, 1980, 1982; Crow, 1983; Crow and Offenbach, 1983).

Retention is generally defined as the period of time post-treatment over which statistically significant differences are detected between experimental and control groups or within experimental groups relative to a pre-treatment response level. Duration of retention varied from 3 to 4 days for conditioned suppression of phototaxis in *Hermissenda* (Crow and Alkon, 1978) to at least 14 to 19 days for modification of feeding behavior in *Pleurobranchaea* (Mpitsos and Davis, 1973) and *Lymnaea* (Alexander *et al.*, 1978). Data on individual differences in acquisition and retention are available for food-aversion learning in *Limax*. Of a sample of 12 animals, 33% retained the aversion for 9 to 26 days after one or two trials; the remaining animals required 3 to 6 trials before retention reached significance (Gelperin, 1975). Mean retention of two non-associative forms of learning, habituation and sensitization of the siphon- and gill-withdrawal reflexes in *Aplysia*, approximated 21 days (Carew *et al.*, 1972; Pinsker *et al.*, 1973). However, because the relationship between training procedures and persistence of the learned response has not been systematically explored in any of these species, these retention periods should be regarded as approximate.

Significant increases in latency, defined as the time an individual *Hermissenda* takes to respond to light, have previously been shown to be specific to temporal pairing of light and rotational stimuli, and to be specifically restricted to locomotion in a light gradient (Crow and Alkon, 1978; Farley and Alkon, 1982; Crow and Offenbach, 1983). Because exposure to paired stimulation results in a decrease in an animal's responsiveness to light, we refer to this learned behavioral change as 'associatively suppressed phototaxis.' Here we report the results of experiments designed to define the range of variation in acquisition and retention of associatively suppressed phototaxis between individual specimens of *Hermissenda*, and the effect of increasing numbers of treatment sessions on retention.

We are interested in describing individual variation in acquisition and retention of this behavioral change for the following reasons. First, the small numbers of neurons in the sensory structures (eye and statocyst) which transduce light and gravitational stimuli, and in the interconnecting sensory pathways, have permitted cellular processes associated with this behavioral change to be analyzed in single neurons (see review by Alkon, 1980; also, Alkon, 1982–1983). To adequately measure changes, often small in magnitude, in membrane currents (Alkon *et al.*, 1982; Farley *et al.*, 1984; Forman *et al.*, 1984) and protein phosphorylation (Neary *et al.*, 1981) that are specific to treatment with paired stimulation, it is important to optimize treatment procedures to induce maximum expression of the learned behavior. Second, variation in retention may itself be correlated with measurable biophysical and biochemical changes. Such correlation, if found, would aid in relating specific cellular processes to behavioral features of learning. Third, since laboratory-reared animals are capable of acquiring the association (Crow and Harrigan, 1979), selective cultivation of strains of animals with long or short retention capacity, as defined by the range in retention measured in the laboratory, could also provide material for study of the behavioral, biochemical, and biophysical components of associative learning.

Materials and Methods

Specimens of *Hermissenda* were obtained weekly, year-round, from Sea Life Supply, Sand City, California, and maintained at 12–14°C in a refrigerated aquarium (Dayno Mfg. Co.). Two fluorescent bright sticks (Sylvania Corp.) provided illumination on a cycle of 12 hours light:12 hours dark (on at 0600) at an intensity of 3.6×10^3 ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} (Radiometer Model 65A, Yellow Springs Instrument Co.). Animals were stored in the aquarium in individually numbered clear plastic slotted containers. All animals were acclimated in the laboratory 4–5 days before the start of an experiment.

Responsiveness to a light gradient was markedly affected by food consumption. Well-fed animals tended to be less responsive to light than semi-starved animals. To ensure survival of animals for long-term (one month) experiments and to control for the effects of food intake on positive phototaxis, it was necessary to standardize feeding so that each animal continued to grow but was not satiated at the time of testing. The feeding schedule selected, by trial-and-error, was 0.10 cm^3 of tunicate viscera (*Ciona intestinalis*) per animal per day, fed at the end of each day's session. This maintenance diet was doubled on two out of seven days for animals larger than 5 cm body length.

Body length of each animal was measured when the animal was fully extended and moving forward. All animals measured 1.50–3.70 cm at the start of an experiment. Sizes were remeasured after 31 days for the group of test-only animals.

Experimental procedures and apparatus have been described by Crow and Alkon (1978) and Tyndale and Crow (1979). Behavioral procedures were divided into two modes, testing and treatment. In the testing mode an animal was secured by a clear plastic gate at one end of a sea water filled clear lucite tube measuring 230 mm by 13 mm (inside diameter) (Fig. 1). Ten tubes were attached to a horizontal turntable, animals at the periphery, in an incubator at 12–14°C. Animals were dark-adapted 10 minutes. The gates were then removed in a darkened room and a light above the turntable center turned on. The latency, or time taken by each animal to move from the dim periphery to the brighter central area was recorded. The turntable did not rotate during testing. In the treatment mode animals were exposed to programmed sequences of 30 seconds of light and 30 seconds of rotation. The rotational stimulus was generated by spinning the turntable. Light and rotation stimulus presentations were either completely paired, unpaired, or randomized (Table 1). In the treatment mode animals remained confined at the turntable periphery, where they were exposed to a gravitational force of $g = 2.24$ during rotation. Latency scores obtained during testing, which preceded and followed treatment, were analyzed to determine the degree to which paired, unpaired, or random light and rotation stimulus presentations affected the animals' responsiveness to the light gradient.

For the present series of experiments illumination conditions were standardized as follows. Light was provided by a series of 150-watt tungsten-halogen lamps (Sylvania Corp. No. EKE) housed outside the incubator (Dolan-Jenner Fiberlite, Model 180). Output from the lamps was combined in branching fiber light pipes of 6.5 mm diameter (Dolan-Jenner Industries) and filtered to 500 $\pm$ 25 nm (green) through a 25.8 cm^2 glass filter (Oriel Corp. No. 5756). The filtered light source was mounted 49.5 cm normal to the turntable center. Peak transmittance of this filter is near peak sensitivity of the photoreceptors as determined by intracellular recordings (510 nm, Alkon and Fuortes, unpub. obs.). In the testing mode, animals experienced a maximum illumination of 2.5×10^3 ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} at the center, decreasing to approximately 2×10^2 ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} at the periphery. In the treatment mode light

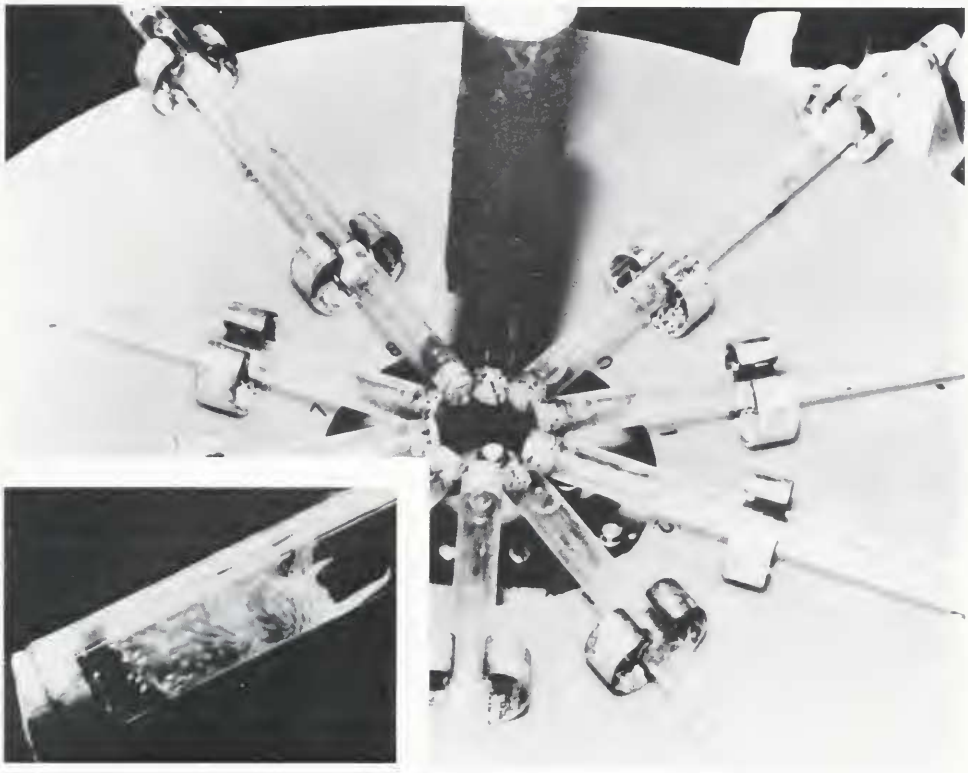

FIGURE 1. Apparatus used for measuring animals' latencies to respond to light (testing), and for treatment with paired, unpaired, or random light and rotation stimulus configurations. Light source is normal to the turntable center; inset shows an animal in the starting position (from Crow and Alkon, 1978).

intensity was increased through the same filter so that animals received a maximum of 2.5×10^3 ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} at the periphery. That is, treatment light intensity equalled test light intensity at the turntable center. Light intensity within the 500 $\pm$ 25 nm band emitted by the aquarium maintenance lights was less than 10 ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}.

Experimental protocol

Only undamaged animals which fed in the laboratory and responded to light within 30 minutes on the initial, or baseline test, were included in experiments. Within each sample of ten animals tested, only three or fewer typically failed to respond. All tests subsequent to baseline response measurement were cut off at 60 minutes. Latencies were recorded by manually activating event recorder pens wired to switches mounted outside the incubator. The response criterion was that the anterior end of the animal, initially one or both tentacles, make physical contact with the plate covering the central end of the tube. Because animals were clearly visible in the experimental green light, minimal uncertainty was involved in this decision. To check possible bias, latency measurements of a sample of ten animals were taken simultaneously by two different experimenters. Recorded latencies were

TABLE I

Experimental design: twenty animals per treatment group were subjected to each of the three stimulus configurations listed (paired, unpaired, or randomized) for 2, 3, 4, and 6 days for each configuration

Treatment	Stimulus configuration	No. of treatment days (100 trials/day)	No. of animals per treatment
Paired L and R	30 s LR on–90 s off (1 trial = 120 s)	2	20
		3	20
		4	20
		6	20
Unpaired L and R	30 s L on–30 s off 30 s R on–30 s off (1 trial = 120 s)	2	20
		3	20
		4	20
		6	20
Randomized L and R	30 s L on–0 to 240 s off 30 s R on–0 to 240 s off (1 trial = variable to 270 s for each stimulus)	4	20
Test-only	—	—	20 Total animals = 200

In the random treatment, stimulus intervals were independently randomized, resulting in partial stimulus overlaps of 20–25%, with about 5 complete pairings occurring per 100 trials.

L = light; R = rotation.

within 0.1 minute per animal. This check was occasionally repeated with a second set of experimenters with the same result.

Within 30–45 minutes after baseline testing, animals were transferred for the first session of light-rotation trials to identical tubes containing clean sea water, dark-adapted for 10 minutes, then subjected to one of the treatments listed in Table I. Because we were interested in maximizing suppression of phototaxis, all sessions were 100 trials each, double the number originally shown to produce significant suppression (Crow and Alkon, 1978). A 60-minute latency test preceded each treatment session. Latencies were also measured in all groups 24-hours post-treatment (the first post-treatment test) on each of the next four days, and once per two days thereafter until each individual's latency recovered to the population median pre-treatment level. All daily latency tests were conducted at approximate 24-hour intervals. Latencies for individual animals were therefore recorded from a baseline value across all treatment days, then until response recovery was observed.

Because the extensive testing and treatment schedule precluded running control and experimental groups simultaneously, effects of variation between shipments and seasonsal effects were controlled for by alternating paired and control treatments and by insuring that each treatment group contained animals from at least three different shipments.

In order to exclude possible effects of habituation and sensitization of phototaxis that might arise over several weeks of repeated testing of the same animal, it was first necessary to assess stability of latencies to light in naive animals over a one month period. An initial group of 10 animals was tested daily for 11 days, simulating 6 treatment days and 5 retention days, then once per 2 days until day 31, simulating a maximum retention of phototactic suppression of 25 days. Choice of an estimated maximum retention period of 25 days was based on results from other gastropods

(see Introduction). During the study similar data were obtained from ten additional animals. These 20 animals formed the test-only group (Table I).

A repeatedly tested group of animals sometimes included individuals which became unresponsive to light within the first 14 days and died within a month thereafter. Because the learned behavior is expressed as a decreased or absent response (to light), care was taken to exclude animals that may have become slow to respond due to a disease process. Therefore, data is reported only from test-only animals which survived in the laboratory at least two weeks after the test period ended.

Because cut-off scores were included in the data, statistical tests were primarily non-parametric (Siegel, 1956; Hollander and Wolfe, 1973). Two forms of latency scores were analyzed. First, all within-group differences and correlations were tested using raw scores in minutes. For the test-only group and for assessment of retention in individuals, raw scores were converted to suppression ratio (SR) scores of the form $A/(A + B)$, where A = baseline latency, B = latency on any subsequent test. A score of 0.50 indicates that baseline and subsequent test latencies were equal; lower scores mean that test latencies have slowed relative to baseline latencies.

Definitions

We define the terms 'acquisition' and 'retention' as they apply to our description of the results as follows.

Acquisition: a statistically significant trend of increasing latencies to light as a function of two, three, four, or six consecutive treatment sessions. For each treatment group, latencies (in minutes) were arrayed from baseline values across latencies from the daily tests preceding each treatment session to the results of the first post-treatment test. This data was analyzed using Page's L-statistic for ordered alternatives, a non-parametric test useful for detecting trends in treatment effects. A value of L was calculated from latency scores ranked within each paired treatment group and control group and its level of significance determined.

Retention: retention of suppressed phototaxis in an individual is defined as the number of consecutive days post-treatment, beginning with the first post-treatment test, on which an animal's suppression ratio (SR) score was less than a median latency score derived from the frequency distribution of SR scores from the test-only group. Retention day scores were determined in this manner for both paired and control group animals.

Results

Stability of phototaxis in the test-only group

Although within-individual latencies varied considerably on successive days, daily median SR scores calculated for the sample were stable over the 31-day test period. When tested against a time trend (days), these scores seemed to decrease with time, but not significantly (Theil test, $C^* = 1.46$, $P = 0.07$, one-tailed). Median scores for individuals over the test period were all ≥ 0.40.

The median score from the frequency distribution of all SR scores combined from this group, with 95% confidence limits, was SR = 0.44 (0.41–0.45). Distribution of scores from the first one-third of the test period (days 2–11, consecutive daily tests) was not significantly different from that obtained over the second two-thirds (days 13–31, tests once/2 days) (Chi square = 6.95, df = 8, $P \leq 0.46$, Fig. 2). Because we did not detect any significant changes in latency to light in this group

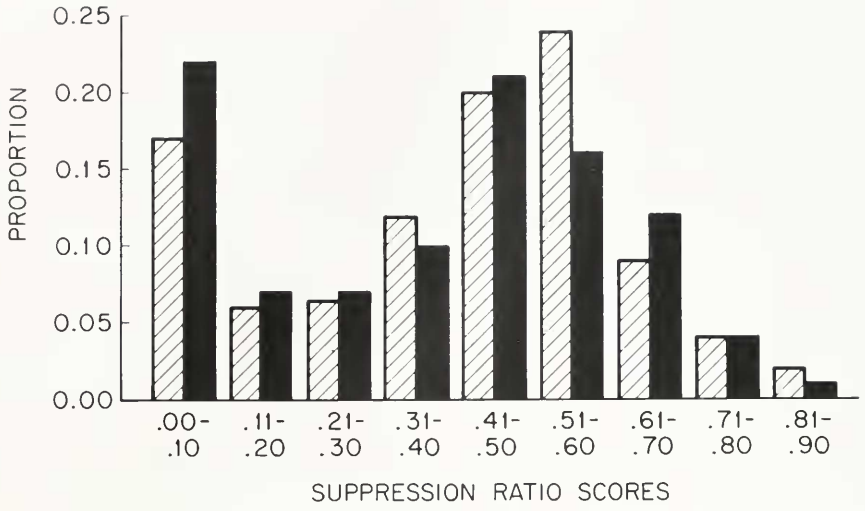

FIGURE 2. Frequency distributions of suppression ratio (SR) scores from the test-only group across days 2–11 (striped bars, 10 tests, n = 200 scores), and across days 13–31 (shaded bars, 10 tests, n = 200 scores). Median scores and 95% confidence intervals for each distribution are SR = 0.45 (0.42–0.48) for days 2–11, and SR = 0.42 (0.40–0.45) for days 13–31. The frequency class 0–0.10 consists entirely of cut-off scores.

over 31 days, we defined the lower 95% confidence limit of the frequency distribution of all scores combined, SR = 0.41, as a conservative score representing the average response obtained from a naive animal tested repeatedly over 31 days. This score is also the entering score for the modal class in the combined distribution, the 0.41–0.50 class. The number of consecutive tests in which an animal's SR score remained below this expected median value could, therefore, represent either a spontaneous run of increased latencies in a control treatment animal, or retention of associatively suppressed phototaxis in a paired treatment animal. Runs of increased latencies to light were considered to be extinguished whenever an individual achieved a test score ≥ 0.41.

Next, we summarized the distribution of runs of increased latencies (scores < 0.41) in the test-only individuals (Fig. 3). The most active animal scored <0.41 on 2/20 test days; at the other extreme one animal had a seven-day run of increased latencies. Seventy-five percent of the runs of suppressed phototaxis in this group were one or two test days in length (Fig. 3). The probability of a spontaneous run of scores < 0.41 for as long as seven days was 1/20 animals, or $P = 0.05$.

There was no significant correlation between baseline latencies in minutes (18/20 animals responded in less than seven minutes), and the total number of days on which each animal scored <0.41 ($r = -0.201$, $df = 18$, $P > 0.05$). Animals slower to respond to light in the baseline test were no more likely than initially faster animals to score <0.41 on repeated tests.

Because the trend (not significant) toward decreasing median latencies across days could have been a function of growth, we compared body lengths measured at the start and end of the 31-day test period. Increase in body length was significant, from 2.32 ± 0.59 cm to 3.54 ± 0.71 cm ($t_{38} = 2.1617$, $P < 0.05$). However, size was not correlated with latency measured on the same day either on day 1 ($r = 0.267$, $df = 18$, $P > 0.05$) or on day 31 ($r = 0.351$, $df = 18$, $P > 0.05$) of testing,

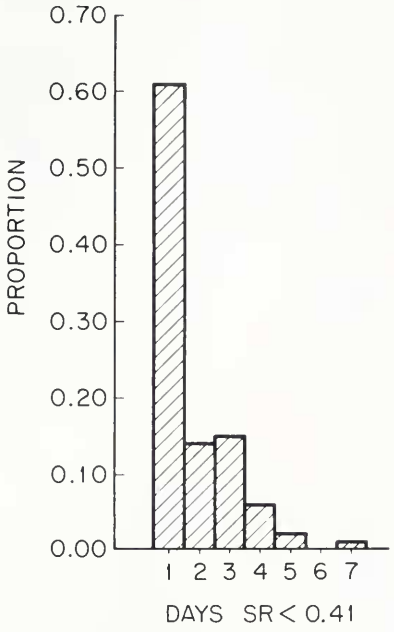

FIGURE 3. Frequency distribution of runs of spontaneously suppressed phototaxis in the test-only group. All animals scored <0.41 at least once in 20 tests over 31 days.

indicating that larger animals in the sample were not consistently slower or faster than the smaller ones. Factors influencing latencies in naive animals across time were not identified in this study.

We conclude that positive phototaxis in *Hermissenda* does not significantly habituate or sensitize over one month of testing and that, within the limits reported, latencies are not a significant function of body size. Prolonged periods of reduced responsiveness to light in animals treated with paired stimulation may, when compared statistically to spontaneously occurring runs of suppressed phototaxis in control treatment animals, be assigned to long-term retention of associatively suppressed phototaxis.

Acquisition

Ninety percent of all experimental animals responded to light within ten minutes in the baseline test. When baseline latencies were compared between all groups no significant difference was found (Kruskal-Wallis one-way ANOVA, $H' = 13.578$, df $= 9$, $P \sim 0.14$).

Although results from the test-only group showed that latencies fluctuate on a daily basis, exposure of animals to paired stimulation should, if acquisition increases with experience, result in a trend towards increasing latencies with number of sessions in the paired but not the unpaired and random groups (Table II, Fig 4).

Arrays of within-group latencies, measured daily from baseline scores across treatment days to the first post-treatment test, were analyzed with Page's L-test for ordered alternatives. All groups (two, three, four, six days) exposed to paired light and rotation showed significant ordered increases in latency across treatment sessions ($P \leq 0.05$). Page's L-statistic did not reach significance (at the 0.05 level) in any

TABLE II

Median latencies in minutes, corresponding median SR scores, and number of animals per group with cut-off scores listed for all treatments from baseline test to first post-treatment test

Paired treatment groups:				Unpaired and random treatment groups:			
	Median latency (min)	Median SR	No. cut-off scores		Median latency (min)	Median SR	No. cut-off scores
2 days				*2 days*			
Baseline	3.8	—	0	Baseline	4.2	—	0
Day 2	4.3	0.47	4	Day 2	3.6	0.54	7
Day 3	5.1	0.43	5	Day 3	3.2	0.57	1
3 days				*3 days*			
Baseline	4.0	—	0	Baseline	2.4	—	0
Day 2	6.4	0.38	2	Day 2	3.4	0.41	3
Day 3	5.7	0.41	6	Day 3	2.6	0.48	1
Day 4	15.2	0.21	6	Day 4	3.4	0.41	2
4 days				*4 days*			
Baseline	2.6	—	0	Baseline	3.3	—	0
Day 2	5.4	0.32	4	Day 2	2.4	0.58	5
Day 3	5.1	0.34	4	Day 3	7.4	0.31	4
Day 4	60+	0.04	11	Day 4	3.4	0.49	3
Day 5	60+	0.04	12	Day 5	4.1	0.45	1
6 days				*6 days*			
Baseline	2.9	—	0	Baseline	3.2	—	0
Day 2	3.8	0.43	6	Day 2	3.2	0.50	3
Day 3	9.9	0.23	8	Day 3	2.4	0.57	2
Day 4	60+	0.05	13	Day 4	3.4	0.48	3
Day 5	60+	0.05	10	Day 5	3.2	0.50	3
Day 6	60+	0.05	11	Day 6	3.7	0.46	3
Day 7	60+	0.05	14	Day 7	3.0	0.52	1
				Random:			
				4 days			
				Baseline	3.0	—	0
				Day 2	7.4	0.26	9
				Day 3	4.7	0.39	3
				Day 4	12.8	0.19	8
				Day 5	5.5	0.35	3
				*Unpaired**			
*1 day**				*1 day*			
Baseline	3.4	—	0	Baseline	3.0	—	0
Day 2	4.3	0.44	16	Day 2	3.0	0.50	17

* *1 day* scores are from the 2 to 6 day treatment groups combined (n = 80). Other values are for independent n = 20 treatment groups.

group subjected to unpaired or random stimulation. Within all paired treatment groups, animals responded significantly more slowly to light on the first post-treatment test than during baseline testing (Wilcoxon matched-pairs signed-ranks test, $P \leq 0.05$, one-tailed). Unpaired and random groups did not show a significantly slower response to light when their first post-treatment test scores were compared to baseline latencies (Wilcoxon matched-pairs signed-ranks test, $P > 0.05$, one-tailed).

Although a separate group exposed to a single treatment session of 100 trials was not run, latencies measured 24 hours after the first session were available from

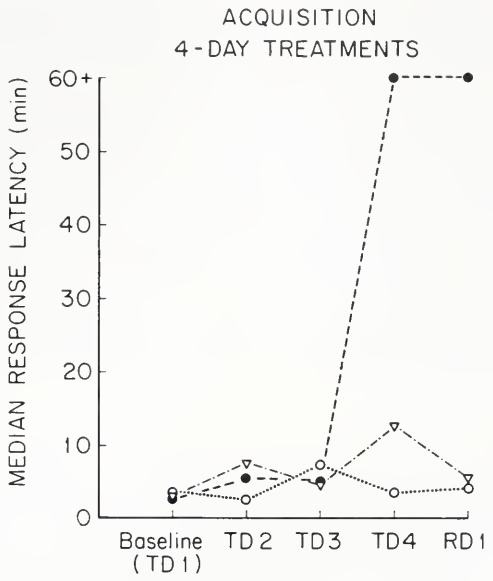

FIGURE 4. Relationship between median response latency in minutes and number of treatment sessions during acquisition in the 4-day groups. Animals were tested before each 100-trial session on treatment days (TD) 1–4. Retention day 1 (RD1) is the first post-treatment test. Paired treatment ●– – –●; unpaired treatment ○– – –○; random treatment ▽—·—▽. Note rapid increase in median response latency of the paired group after three treatment sessions.

all groups. Latencies were significantly slower than baseline values 24-hours after one paired treatment session (all paired treatment groups combined, Wilcoxon matched-pairs signed-ranks test, $n = 77$, $P = 0.002$, one-tailed). No significant increases in latency were detected between latencies measured before and 24-hours after any control treatment (all unpaired groups combined, Wilcoxon matched-pairs, signed ranks test, $n = 78$, $P = 0.32$, one-tailed; one random group, Wilcoxon test, $n = 20$, $P > 0.05$).

These results demonstrate that acquisition of the behavioral change, or increase in latency to respond to light, may be detected 24 hours after exposure to at least 100 trials with paired light and rotation. However, the increase in within-group median latency measured after one 100-trial session is small, approximately one minute (Table II), and large sample sizes of $n \geq 80$ may be necessary in order to consistently measure a 24-hour associative effect. Latency increases within paired groups were consistently significant with sample sizes of $n = 20$ when training sessions spanned two or more days. Trends in latency scores measured 24 hours after each unpaired or random treatment session were not significant even after six consecutive sessions.

Inspection of latency scores from the first post-treatment tests from all paired treatment groups indicated that the magnitude of the change in latency increased with the number of sessions. As the number of paired treatment sessions increased from two to six, the number of animals scoring <0.41 on the first post-treatment test increased. Also, a discontinuity was observed in the latency score distributions that separated results of two and three treatment sessions from those of four and six sessions (Table II). Numbers of non-responding animals, that is, those that did not traverse the light gradient within 60 minutes and were given cut-off scores,

counted within each group were five and six on the first post-treatment test for animals trained two and three days, increasing to 12 and 14 for animals trained four and six days. Within unpaired and random groups, on the first post-treatment test, numbers of non-responding animals did not increase across sessions (Table II).

Next, within each paired-treatment group, latencies for those animals that responded to light before the 60-minute cut-off were compared with their baseline latencies. These distributions were not significantly different within the two or three day groups, but reached significance within the four and six day groups combined [Wilcoxon matched-pairs, signed-ranks test, one-tailed: n = 15, $P > 0.05$ (two days); n = 13, $P > 0.05$ (three days); n = 12, $P = 0.025$ (four and six days)]. These results suggest that individuals differ in sensitivity to the learning paradigm, with less sensitive animals showing significant behavioral suppression only after four or six paired treatment sessions.

Retention

The discontinuity on the first post-treatment test between numbers of non-responding animals (within the 60-minute test) from paired groups treated two and three days *versus* four and six days is reflected in the retention day score distributions (post-treatment runs of SR scores < 0.41). Animals subjected to paired light and rotation for four or six days had a significantly broader distribution of retention day scores than did the two and three day groups (Table III, Fig. 5A, B). Retention day scores were bimodally distributed in the four-day paired group; 14/20 animals scored 0–4 retention days and 6/20 scored 11–14 days. The distribution was smoother after six treatment days (Fig. 5A). Maximum retention was measured in the six-day group at 17–18 days (one animal). As the number of sessions increased both the number of animals scoring at least one retention day and the maximum number of retention days increased relative to the distribution of similarly determined runs of suppressed phototaxis in the unpaired and random groups (Fig. 5A, B).

TABLE III

Tests of significance between retention day distributions

Comparison	Statistic
Unpaired groups on 2, 3, 4, 6 treatment days and 4-day random group	H′ = 2.522, df = 4 (NS)
Paired groups on 2, 3, 4, 6 treatment days	H′ = 22.643, df = 3, $P \leq 0.01$ (by Miller's multiple treatment comparisons: days 4, 6, sig. dif. ($P \leq 0.05$) from days 2, 3)
2 days: paired *versus* unpaired	D_{max} = 3 (NS)
3 days: paired *versus* unpaired	D_{max} = 6 (NS)
4 days: paired *versus* unpaired	D_{max} = 8 ($P \leq 0.05$)
4 days: paired *versus* random	D_{max} = 9 ($P \leq 0.05$)
6 days: paired *versus* unpaired	D_{max} = 12 ($P \leq 0.01$)

H′ = Kruskal-Wallis statistic (one-way ANOVA by ranks).
D_{max} = Kolmogorov-Smirnov statistic (test for broad alternatives).
All tests are one-sided.
NS = not significant at $P \leq 0.05$.

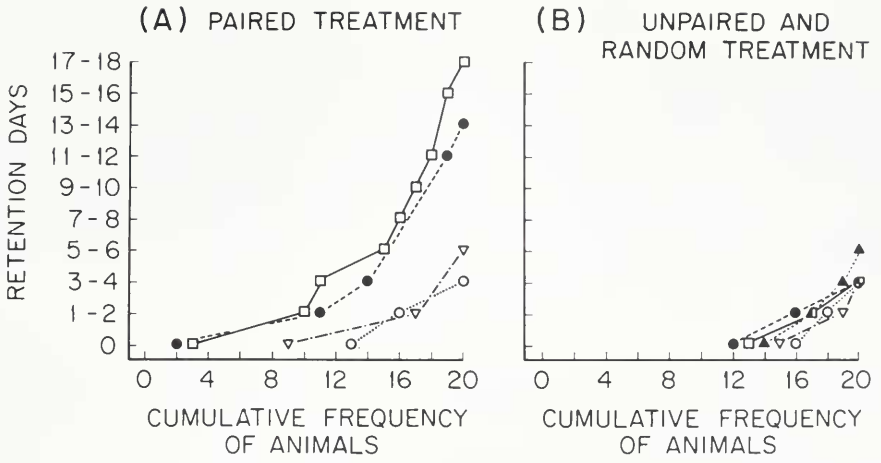

FIGURE 5. Relationship between retention day score distributions and number of treatment sessions, expressed as cumulative frequency of animals across retention day score classes. (A) Effect of paired treatments. Note the discontinuous distribution of retention days between the 2- and 3-day paired groups and the 4- and 6-day groups. (B) Effect of unpaired and random treatments. Distributions of post-treatment runs of spontaneously suppressed phototaxis for these groups. Note that more than half of each control group consisted of animals scoring zero retention days. Two days ○ - - - ○; 3 days ▽ —·— ▽; 4 days ● - - - ●; 6 days □ —— □; 4-day random group ▲ - - - ▲.

Significant increases in retention in paired relative to control groups were detected only for the four and six day groups (Table III). There was no significant correlation between baseline latencies of animals in the four and six day paired groups and their retention day scores ($r = -0.0563$, $df = 38$, $P > 0.05$).

If animals scoring more than seven post-treatment retention days, scores unique to the paired treatment (Fig. 5A) and likely to occur with a probability of less than 0.05 (see results from the test-only group), are defined as 'long-retainers,' then 11/40 (27.5%) of the four plus six day paired groups may estimate that fraction of the laboratory population capable of long-term retention of associatively suppressed phototaxis.

Can first post-treatment test scores predict retention day scores?

Because the criterion for recovery of responsiveness to light to an average pre-treatment level was expressed in the form of an SR score ($SR \geq 0.41$), latencies were analyzed for their predictive value in this form. SR scores < 0.41 on the first post-treatment test were combined for all paired groups and arranged into four samples of scores for animals that subsequently scored 1, 2, 3–6, and 7+ retention days (medians = 0.28, 0.04, 0.08, and 0.05, respectively). Overall, these scores were significantly different (Kruskal-Wallis one-way layout, $H' = 14.955$, $df = 3$, $P \leq 0.005$). The sample of animals that scored one retention day had significantly higher SR scores on the first post-treatment test than did samples of animals scoring more than one retention day (Dunn's multiple comparison method, $P \leq 0.05$). From inspection of the data, 75% of animals with one retention day ($n = 14$) had SR scores greater than 0.20, whereas among animals scoring more than one retention day ($n = 39$) 75% scored less than SR = 0.20.

A similar analysis of SR scores from the unpaired groups resulted in no significant differences detected between SR scores arranged into three samples of 1,

2, and 3–4 retention day scores ($H' = 0.669$, df = 2, $P \sim 0.70$). Median SR scores for these groups were 0.18, 0.15, and 0.16, respectively.

Discontinuities in behavioral responses between subgroups of paired treatment animals, such as differences in retention measured between animals scoring above or below SR = 0.20 on the first post-treatment test or the bimodal distribution of retention days seen in the four-day paired treatment group, suggest that data on cellular events taken from animals within different behavioral subgroups be compared to see if the behavioral differences are reflected in biophysical and/or biochemical differences.

Discussion

Our data suggest that the extensive variation in retention observed among animals exposed to paired stimulation with light and rotation for four and six daily sessions may represent differences in individual capacities for associative learning. A factor that may contribute significantly to this variability is the level of food intake of the animals. Latency measurements obtained while determining an optimum feeding level for the experimental animals indicate that animals fed to excess (more than they would consume) tended to respond more slowly to light and with greater variability than did semi-starved animals.

Even greater variation between individuals may have been detected if experimental animals had not been selected for uniformly fast responsiveness to the light gradient before treatment. Most animals tested (70–100% of each sample of ten) were likely to have baseline latencies less than 30 minutes. Those animals with initially longer latencies (30–60+ minutes; not included in present study), when retested on a later day, either responded faster, equally slowly, or were unresponsive to the test light over ten or more test days (J. Harrigan, pers. obs.). Results reported here were obtained only from animals capable of a strong photopositive response during baseline testing, and may not be applicable to the small proportion of the population less responsive to light gradients.

Selection of initially fast animals, 90% with latencies less than ten minutes, also reduced the possibility of including in experiments animals whose latencies may have been suppressed by recent experience with paired light and gravitational stimulation in the ocean, or animals which did not recover from collection and shipment. Elapsed time between field collection and the start of an experiment was approximately 9–11 days. Our results indicate that only 27% of experimental animals (the four plus six day paired treatments) exhibited suppressed phototaxis for seven or more days, and that increased latencies during this period were usually in excess of 60 minutes (cut-off scores). However, when working with animals from wild populations the influence of prior experience and the effect of behavioral 'savings' (Crow and Alkon, 1978) on subsequent experiments on learning cannot be completely ruled out.

In previous investigations, animals were trained with light and rotation on a schedule consisting of 50 trials per day for three days. Routinely, statistically significant latency increases were found using either within-group tests or comparisons with the latencies of control groups. Previously published values for mean and median latency scores for paired groups, expressed as minutes or SR scores, are similar to values reported here for three days of training with 100 trials per day. Crow and Alkon (1978) report a median SR score of 0.30 for a paired treatment group 48 hours post-treatment with 150 trials over three days (present study = 0.21

SR; at 24 hours post-treatment, Table II). Farley and Alkon (1982) present two figures (Figs. 1, 2, Farley and Alkon, 1982) comparing mean latencies in minutes and SR scores for groups of paired treatment animals in a horizontal light gradient. They report mean baseline latencies of approximately 9–13 minutes (present study = 2.6–4.2 minutes, Table II), and 24 hours post-treatment latencies of about 24–28 minutes, or SR about 0.30–0.35 (present study = 15.2 minutes, SR = 0.21, Table II). Mean increase in latency between the baseline and first post-treatment tests was estimated at 15 minutes in Farley and Alkon's experiment, and 11 minutes in the present study. Longer latencies in the baseline test in Farley and Alkon's experiment may reflect differences in maintenance and in experimental lighting conditions; however, the mean latency increase after 150 trials was nearly the same as that reported here for 300 trials over three days.

These results suggest an interaction between treatment days and trial density during acquisition. Latency measurements for all paired treatment groups in our study show an average increase of 1.3–7.0 min between baseline and day three tests (after two training days), increasing to 11.2–60+ minutes after three training days (Table II). We found consistently large increases in mean latency (to 60+ minutes) mainly in the groups trained four and six days; shorter training schedules were associated with increased variability between groups (Table II). Although significant acquisition was measured in all paired-treatment groups, a significant increase in the number of days that the association was retained was measured only in groups trained more than three days (Table III).

Retention may also be affected by trial density. Longer retention of associative learning after spaced rather than massed trials has been demonstrated in *Lymnea* (Alexander *et al.*, 1982), which is also capable of significant acquisition after one trial (Alexander *et al.*, 1984). Retention of habituation and sensitization of the gill- and siphon-withdrawal reflex in *Aplysia*, two non-associative forms of learning, were also significantly enhanced by spaced rather than massed trials (Carew *et al.*, 1972; Pinsker *et al.*, 1973). Influence of trial density on retention in *Hermissenda* has not yet been quantified.

Each post-treatment test can also be considered a measure of resistance to extinction. Retention of suppressed phototaxis was surprisingly persistent considering the frequency with which latencies were measured. Longer periods of suppression may possibly have been measured with a less frequent testing schedule. Also, because the presence of learning in *Hermissenda* was expressed as a reduction in responsiveness (to light), an animal could be assigned a retention day score only when its latency recovered to the average score for the naive population, SR $\geq$ 0.41. It is possible that some animals were 'permanently' trained, and that retention exceeding 18 days was missed because animals died before recovering to SR $>$ 0.41 and were therefore excluded from the data.

Associatively suppressed phototaxis has been demonstrated in laboratory-reared *Hermissenda*. Three consecutive generations of animals reared from wild parents (the F_1, F_2, and F_3 generations) acquired the behavioral change after three days of training with 50 trials per day of paired light and rotation (Crow and Harrigan, 1979). These cultured populations showed significantly less variation in their latencies to respond to light than did animals from wild populations (Crow and Harrigan, 1979). It is not known if retention day scores would also be less variable in laboratory-reared animals. Along with possible heritable components of learning, the ability of an individual *Hermissenda* to acquire this associative task is also influenced by environmentally induced alterations in sensory system morphology

that occur during larval and juvenile development (Crow and Harrigan, 1979; Harrigan, Crow, Kuzirian, and Alkon, in prep.).

In a review of definitions of learning as they might apply to *Pleurobranchaea*, Mpitsos *et al.* (1978) concluded that the effects of different controls on neural functioning must be understood before the one most appropriate for the particular feature of learning under investigation can be selected. Choice of controls for conditioning procedures, especially for initial demonstrations of associative learning, have generally been some combination of naive, unpaired, random, and single stimulus presentations tailored to demonstrate specificity of the particular association under study to the temporal pairing of stimuli. In recent studies on *Hermissenda* combining behavioral and cellular analyses, the control treatment selected has been either random or unpaired. In the random control, partial stimulus overlaps were obtained with separately randomized light and rotation plus a small number (fewer than 5) of complete pairings, providing a conservative control against which cellular events induced by paired stimulation can be assessed. In the unpaired control, it is assumed that no association between stimuli is formed (for example, Crow and Alkon, 1982).

In the present study and other studies on *Hermissenda* in which acquisition was measured 24 hours post-treatment, no significant behavioral differences were found within or between unpaired, random, or single stimulus treatment groups; these controls were behaviorally identical. Because our study included only behavioral data, adequate controls were considered to be unpaired groups corresponding to each paired treatment, and a single random (four-day) control treatment as a check for the presence of any non-associative effects that might have accrued from increasing the number of trials and sessions over those previously used. The small number of complete pairings that occurred during the random treatment (15) had no measurable effect on post-treatment relative to baseline latencies.

In a series of conditioning experiments on *Hermissenda*, Crow (1983) found significant non-associative effects, affecting paired and random treatments equally, 30 minutes after exposure to 50 paired trials with light and rotation. These non-associative effects decremented by 45 minutes post-training when paired and random groups became significantly different, reflecting the appearance of longer-term associative effects and did not accumulate over multiple training sessions (Crow, 1983). Non-associative effects, therefore, were not detectable with the 24-hour interval employed in the present study.

The behavioral change is pairing-specific and affects phototaxis in both vertical and horizontal planes (Crow and Alkon, 1978; Farley and Alkon, 1982). Animals have been observed, after training, to move around in non-gradient illumination and feed normally (J. Harrigan, pers. obs.), indicating that only orientation components have been affected. Because strong positive phototaxis appears primarily in semi-starved animals (Alkon *et al.*, 1978; present study), Alkon (1980) has suggested that phototactic suppression may enhance survival in the natural habitat by inhibiting migration from depleted food supplies into brightly lit surface waters during strong surge, which the rotational stimulus mimics.

Hypotheses regarding possible adaptive advantages of conditioned phototactic suppression and its behavioral variability will have to be tested in the field rather than the laboratory. Differences in patterns of light and gravitational stimulation between the laboratory and the animals' natural habitat, as well as differences in the stimulus parameters themselves, especially the rotational stimulus employed in the laboratory, preclude any generalizations from behavioral results obtained in the laboratory to naturally occurring behavior patterns.

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